High-performance thin-layer chromatographic resolution of oligogalacturonic acids

Citrus pectin-derived D-galacturonan (polygalacturonic acid, PGA) preparations are commercially available. Partial hydrolysis of PGA with polygalacturonanases yields a mixture of oligogalacturonic acids, from which individual oligomers can be chromatographically isolated. To monitor such separations in efforts to isolate gram quantities of pure oligogalacturonic acids, we needed an effective t.l.c. procedure. Oligomers up to d.p. 6 have been resolved on plates of silica gel by single development with a mobile phase[1]. On plates of microcrystalline cellulose, mixtures up to d.p. 9 were resolved, but three developments were required[2]. In both cases, separations were conducted on 20-cm plates, and a high level of an organic acid was present in the mobile phase, so that irrigation times were high. We describe here some rapid separations of oligogalacturonic acids up to d.p. 9 on high-performance t.l.c. plates (10 × 10 cm) of silica gel, using single developments with ethanol–aqueous acetic acid mobile phases.

High-performance (h.p.) t.l.c. plates of silica gel (Type HP-K, Whatman) were used, and comparable separations were not achieved with other plates. The mixture of oligogalacturonic acids was prepared by partial hydrolysis of PGA by Sigma "pectinase", a preparation having activities additional to those of exo- and endo-polygalacturonanase. There was no evidence for the formation of unsaturated products by the action of pectic acid lyase. The h.p.t.l.c. profile of the ethanol precipitated, partial hydrolyzate is shown (lane d) in Fig. 1. The mobile phase was 21:29 ethanol–25mM acetic acid, and the plate was irrigated at 35°. Also spotted were galacturonic acid (lane a) and partially purified oligogalacturonic acids having d.p. values of 2 and 3 (lanes b and c) and 4–6 (lanes e, g, and f). These standards were isolated by gradient elution (sodium formate, pH 4.7) of the PGA partial hydrolyzate from a strong-base anion-exchange resin (formate form). The macroreticular resin AG MP-1 provided a more rapid and more efficient resolution of the oligomers than do the Dowex resins, which have been most commonly used for this

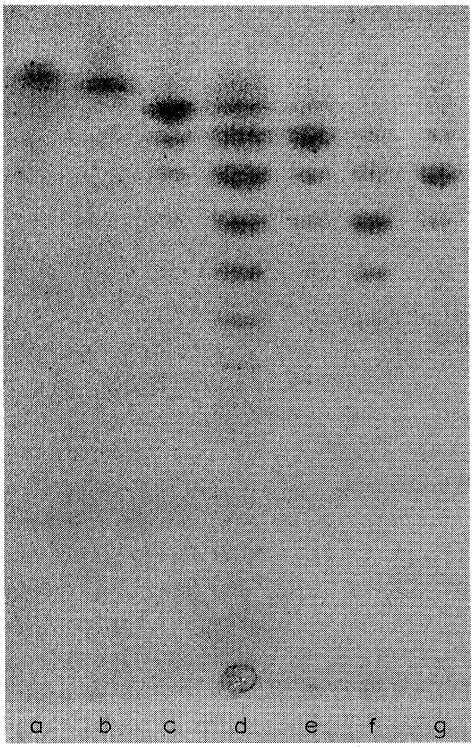

Fig. 1. H.p.t.l.c. of partially purified oligogalacturonic acids and partial "pectinase" hydrolyzate of polygalacturonic acid. Mobile phase, 21:29 ethanol–25mM acetic acid; plate irrigated at 35°. [Key: a, galacturonic acid; b, digalacturonic acid; c, trigalacturonic acid; d, partial "pectinase" hydrolyzate of polygalacturonic acid; e, tetragalacturonic acid; f, hexagalacturonic acid; and g, pentagalacturonic acid.]

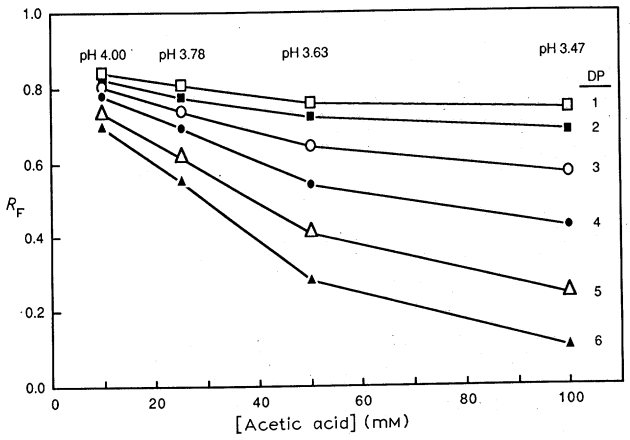

Fig. 2. Relationship between R_F values of individual oligogalacturonic acids and millimolarity of acetic acid in 21:29 ethanol–aqueous acetic acid mobile phases. Plates were irrigated at 24°.

purpose[3]. The d.p. 3, 5, and 7 oligomers were analyzed by fast-atom-bombardment mass spectrometry, and gave the expected molecular-ion masses.

Mobilities of oligogalacturonic acids on type HP-K plates increase with pH of the mobile phase (see Fig. 2). Adjustments of pH from 3.47 to 4.00 were made by decreasing the concentration of acetic acid in the 21:29 ethanol–aqueous acetic acid from 100 to 10mM. The pK values for the individual oligomers are within this pH range, and suppressing the ionization results in decreased mobility. R_F values are more conveniently modified by changing the proportion of ethanol in the mobile phase, and this effect is shown in Fig. 3. Oligomers in the lower d.p. range (1–3) are best separated with a mobile phase consisting of 23:27 ethanol–25mM acetic acid. Changing the ratio to 21:29 provides mobility of components up to d.p. 9 (see Fig. 1). The R_F values in Figs. 2 and 3 were calculated from results obtained by using plates irrigated at 24°. The mobilities of individual oligogalacturonic acids increase with temperature, with R_F values of those of d.p. >5 approximately doubling for each 10° increase in temperature. We recommend that plates be irrigated at 35° when analyzing mixtures, as the components are then more effectively resolved.

Oligogalacturonic acid mixtures of 1–4 μg/μL were applied to the plates, and the aniline–diphenylamine spray reagent[4] provided sensitive spot detection. A total of 7 μg was applied in lane d, Fig. 1. The procedure is not generally applicable to the analysis of oligogalacturonic acids having a d.p. >9. When analyzing column fractions consisting of higher-d.p. mixtures, we have occasionally observed spots representing d.p. 10 and 11, especially when the plates were irrigated twice. Oligogalacturonic acids having a d.p. >6 are minimally soluble in the mobile phase, however, and only a portion migrates up the plate on irrigation. When plates are irrigated twice, a major (lower R_F) and a minor (higher R_F) spot are observed for each of the fragments having d.p. >6. As a result, the procedure is qualitative, and only for fragments of d.p. 1–6 can quantitative estimates be made on the basis of spot intensity.

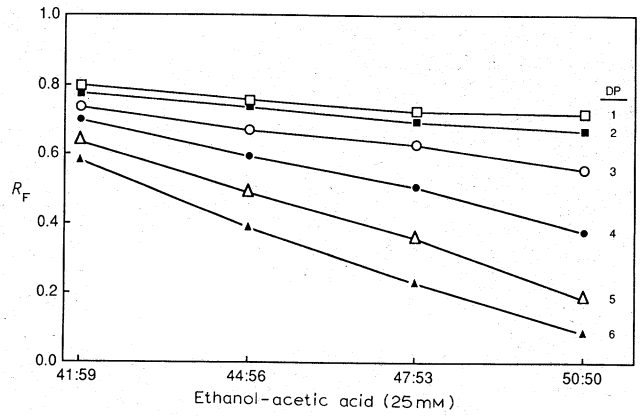

Fig. 3. Relationship between R_F values of individual oligogalacturonic acids and proportion of ethanol in ethanol–25mM acetic acid mixtures. Plates were irrigated at 24°.

To summarize, oligogalacturonic acid mixtures up to d.p. 9 are effectively resolved on type HP-K t.l.c. plates by using a single irrigation with ethanol–25mM acetic acid at 35° (see Fig. 1). Those having d.p. values of 10 and 11 can be detected, if present in sufficient quantities, after an additional irrigation. The separation are accomplished over a path length of 8.5 cm, and each plate development requires 110 min. This h.p.t.l.c. procedure is faster than existing methods for separating oligogalacturonic acids, and provides a much better resolution of individual oligogalacturonic acids.

EXPERIMENTAL

High-performance t.l.c. procedures. — Precoated plates (10 × 10 cm) of silica gel type HP-K (4.5 μM particle size, 60 A pore diameter, 200 μm layer thickness) were purchased from Whatman (Clifton, NJ). These plates were activated for 15 min at 110°, and stored in a desiccator. Mobile phases were added to closed, glass, irrigation chambers each day. After plate irrigation and drying, spots were made visible by spraying with a solution[4] of aniline (4 mL), diphenylamine (4 g), acetone (200 mL), and 85% phosphoric acid (30 mL), and then heating for 20 min at 120°, or placing on a hot plate for a few seconds.

Partial enzymic hydrolysis of PGA. — Sigma PGA (20 g) was stirred into 100mM sodium hydrogencarbonate (800 mL, pH 8.21) over during 15 min, the pH gradually dropping to 4.28. Sigma "pectinase" (3.2 mL, 40% solution in glycerol) was added, and the mixture was then stirred for 3 min the reaction quenched by heating to 80° in a water bath, and the mixture cooled to 40° and filtered through Celite. Ethanol (95%; 2 vol.) was added, and, after stirring 15 min, the precipitated oligogalacturonic acids (sodium salts) were collected by centrifugation at 4000*g*. Much of the galacturonic acid and some digalacturonic acid, which were not needed, were retained in the supernatant liquor. The pellet was stirred in absolute ethanol, filtered, and dried *in vacuo*, yielding oligogalacturonic acid mixture (16.2 g).

Chromatographic isolation, and characterization of partially purified di-, tri-, tetra-, penta-, hexa- and hepta-galacturonic acids. — Separation was achieved by step-gradient elution from Bio-Rad strong-base anion-exchange resin (100–200 mesh) AG MP-1 (formate). To a glass column (2.0 × 40 cm) packed with a slurry of resin (100 mL) in 0.30M sodium formate buffer (pH 4.7) was applied 2.0 g oligogalacturonic acid mixture (sodium salts) in water (10 mL). The column was sequentially eluted (3.5 mL/min) with 132-mL steps of 0.2M, 0.3M, 0.4M, 0.5M, 0.6M, and 0.7M sodium formate buffer (pH 4.7). Fractions (22 mL each) were collected, and their composition monitored by the h.p.t.l.c. procedure described herein. Aliquots (2 μL) from each tube were spotted, and the plates were irrigated at 35° with 21:29 ethanol–25mM acetic acid. Individual oligogalacturonic acids having d.p. values of 2 to 7, and estimated to be in excess of 90% purity (by t.l.c.), were obtained by pooling tubes 8–10, 14–16, 20–22, 23–26, 27–29, and 30–35,

respectively. Addition of 2 volumes of acetone to the fractions resulted in precipitation of the oligogalacturonic acids, and they were collected by centrifugation at 4000*g*. Each pellet was dissolved in water (20 mL), and the solution stirred with Bio-Rad AG-50W X-8 (H^+) resin, to convert the products from the sodium into the free acid form. Fluffy white powders were obtained after removing the resin by filtration, and lyophilizing the filtrates. Yields of 30 (d.p. 2) to 140 (d.p. 5) mg were obtained. Fast-atom-bombardment mass spectra of d.p. 3, 5, and 7 oligogalacturonic acids gave the expected values of 569, 921 and 1273, respectively, for the quasimolecular ions ($M + Na^+$). There was no evidence for the presence of products resulting from pectic acid lyase activity.

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